

Changes in the Bacterial Flora of Skin of Processed Broiler Chickens Washed in Solutions of Salicylic Acid

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Abstract: Changes in the number of bacteria recovered from the skin of processed broilers after each of five consecutive washings in salicylic acid (SA) solutions was examined. Skin samples from commercially processed broiler carcasses were divided into 3 groups and washed in distilled water (control), 10% SA, or 20% SA by agitating skin in wash solutions in a Stomacher laboratory blender. After each wash, skin was transferred to fresh solutions and washing was repeated to provide samples washed 1 to 5 times in each solution. Washed skin was stomached in Butter field's Phosphate Buffer to recover bacteria on the skin. Bacterial flora of the rinsates was enumerated on Plate Count (PC) Agar, *Staphylococcus* (STA) Agar, Levine Eosin Methylene Blue (EMB) Agar, Lactic Acid Bacteria (LAB) Agar, and *Perfringens* (PER) Agar with TSC supplement; and then bacterial isolates from each medium were identified. Results indicated that after each of 5 consecutive washes in water, there was no significant difference in the number of bacteria recovered from skin on any of the agar media. Significantly fewer bacteria were recovered on LAB Agar from skin after 5 washes in 10% SA than after 1 wash, but there was no significant decrease in the number of bacteria recovered on any other media after skin was washed in this solution. However, washing skin 4 or 5 times in 20% SA significantly reduced the number of bacteria recovered on PC and STA Agar, while no bacteria were recovered on EMB or LAB Agars from rinsates of skin washed 4 or 5 times in 20% SA or on PER Agar from skin washed 3 or more in the 20% solution. *In vitro* studies indicated that SA is bactericidal towards bacterial isolates recovered from skin and that resistance to the bactericidal activity of SA in descending order is *Staphylococcus simulans* > *Lactobacillus* > *Escherichia coli* > *Clostridium perfringens*. Findings indicate that successive washing of skin in SA may significantly reduce the number of bacteria recovered from the poultry skin and that the bactericidal activity SA can kill bacteria *in vitro*.

Key words: salicylic acid, poultry skin, bactericide, staphylococci, coliforms

Introduction

The skin of processed broiler chickens consists of a diverse microflora (Cason *et al.*, 2007; Kotula and Pandya, 1995). The microflora includes bacteria that normally inhabit the skin of live, healthy chicken (Thomas and McMeekin, 1980) and bacteria that contaminate the skin during various processing operations (Berrang *et al.*, 2001; Cason *et al.*, 2007). Various chemical sanitizers are used by commercial poultry processors to reduce microbial contamination of poultry carcasses (Keener *et al.*, 2004; Russell and Keener, 2007), but some microorganism can still be recovered from the skin of processed poultry (Northcutt *et al.*, 2003). Microorganisms that colonize the skin of live broilers may be protected from the antimicrobial activity of sanitizers by fats and proteins secreted by the skin (Thomas and McMeekin, 1980). Other bacteria that attach to skin during processing may also escape antimicrobial treatments by inhabiting crevices and folds on the surface of the skin (Lillard, 1986). These bacteria are continuously shed during washing, and it has been previously demonstrated that there is no significant

difference in the number of bacteria recovered from broiler carcasses after successive whole - carcass - rinses (Izat *et al.*, 1991; Mead and Thomas, 1973; Rigby, 1982). Furthermore, aerobic bacteria and Enterobacteriaceae can be recovered from picked carcasses after up to 40 consecutive whole carcasses rinses in peptone water (Lillard, 1989). Consequently, contaminated poultry products continue to be a source of human foodborne diseases (Institution of Food Technologist, 2004; Mead *et al.*, 1999).

Microbicidal surfactants, such as alkaline salts of fatty acids (Hinton and Ingram, 2006) can reduce the number of microorganisms on the surface of processed poultry products (Hinton *et al.*, 2007). The surfactant activity of these compounds may increase the cleansing activity of water, while the microbicidal activity of the substances can kill microorganisms by causing lysis of microbial cellular membranes. However, some bacteria that are susceptible to the antibacterial activity of alkaline salts of fatty acids *in vitro* exhibit a relatively high degree of resistance to these compounds when the bacteria are attached to poultry skin (Hinton *et al.*, 2007). Sanitizers

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that can remove substances that provide protection to microorganisms attached to the skin may be more effective in reducing the number of these organisms on contaminated carcasses.

Salicylic acid (SA) is an organic acid that possesses keratolytic activity that can remove dead layers of the skin by dissolving the cement-like material that holds the cells together (Huber and Christophers, 1977; Shames *et al.*, 1989). Although some of the outer skin layer, as well as many of the bacteria attached to the skin, are removed by scalding and picking operations, part of this skin layer can still be found on the picked carcass (Thomas and McMeekin, 1980). Treatments that can remove a portion of the outer layer of skin cells may eliminate some of the bacteria attached to this layer of skin. The purpose of the present study was to determine if washing skin of processed broiler chickens in solutions of SA would reduce bacterial contamination of the skin.

Materials and Methods

SA solutions: A 20% wt/vol solution of sodium salicylate (Sigma Chemical Co., St. Louis, MO) was prepared in distilled water, and then filter sterilized by passage through 0.2µm filters (Nalge Nunc International, Rochester, NY). Ten percent SA solutions were prepared by combining equal volumes of the 20% solution and sterile distilled water. Sterile distilled water was also used to wash skin that served as control samples.

Determination of the effect of successive washing in SA on the bacterial flora of poultry skin: Broiler carcasses were taken from the processing line of a local, commercial poultry processing facility after exiting the mechanical picker. Carcasses were immediately placed in coolers with crushed ice and transported to the laboratory. Breast skin was removed from carcasses, cut into 5 g pieces, and refrigerated at 4°C until ready for use. All skin samples were used within 5 days of refrigerated storage.

Skin samples were washed by placing skin in sterile stomacher bags; adding 45 ml of sterile distilled water (control), 10% SA, or 20% SA; and stomaching on high speed in a Stomacher 80 (Seward Limited) for 1 min. The washed skin was then transferred to another sterile stomacher bag, and 10 ml of Butterfield's Phosphate-Buffered Dilution Water (Food and Drug Admin/Center for Food Safety and Applied Nutrition, 2001). was added. Bacteria remaining on the skin samples were recovered by stomaching skin in buffer for 1 min on high speed. Skin was discarded, and aliquots of the buffer rinsates were removed for microbial analyses. The washing procedure described above was repeated using separate pieces of skin that were washed by stomaching in fresh 45 ml aliquots of distilled water, 10% SA, or 20% SA for 1, 2, 3, 4, or 5 times. The rinsing procedure was repeated after the final

wash, and an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Bethesda, MD) was used to plate rinsates on agar media. Rinsates were plated on Difco Plate Count (PC) Agar (Becton Dickinson, and Co., Sparks, MD); Staphylococci (STA) Agar, Medium 110 (Oxoid Ltd., Basingstoke, Hampshire, England); Levine Eosin Methylene Blue (EMB) Agar (Remel Inc., Lenexa, KS); Lactic Acid Bacteria (LAB) Agar (Atlas, 1993) and Perfringens (PER) Agar Base with egg emulsion and TSC selective supplement (Oxoid Ltd.). Inoculated PC, STA, and EMB agars were incubated aerobically at 35-37°C for 48 h; and inoculated LAB and PER plates were incubated anaerobically in a Coy Anaerobic Chamber (Coy Laboratory Products Inc., Grass Lake MI) at 35-37°C for 48 h. After incubation, colonies on agar plates were counted using the QCount™ Colony Counting System (Spiral Biotech). Each experiment was repeated 5 times.

Colonies from agar plates that contained isolates recovered from skin samples washed 5 times in distilled water or 20% SA were selected for identification. Isolates recovered on EMB Agars were identified using the BD BBL™ Enterotube™ II kit (Becton Dickinson, and Co.), while isolates from all other media were identified using the MIDI Sherlock Microbial Identification System, Version 6.0 (MIDI, Inc. Newark, DE).

Determination of the bactericidal activity of SA

solutions: Cultures of *Staphylococcus simulans* and *E. coli* were grown aerobically at 35-37°C for 18-24 h in Tryptic Soy Broth (Difco), cultures of *Lactobacillus* were grown anaerobically at 35°C in *Lactobacillus* MRS Broth (Difco), and cultures of *Clostridium perfringens* were grown anaerobically at 35°C in Reinforced *Clostridia* Medium (Difco). After incubation, cultures were centrifuged at 5500g in a Hermle Z300 centrifuge (National Labnet Co., Woodbridge, NJ) for 5 min. Supernatant was discarded, cell pellet was suspended in 10 ml of fresh Butterfields phosphate buffer, and centrifugation was repeated. A suspension of the final pellet was added to an aliquot of Butterfields phosphate buffer to produce an absorbance of 1.00 at 625 nm in a Spectronic® 20D+ spectrophotometer (Spectronic Instruments, Inc. Rochester, NY). One tenth ml of the bacterial suspension was added to test tubes containing 9.9 ml of distilled water, 10% SA, or 20% SA; and tubes were shaken for 1 min using a Mistral Multimixer (Lab Line Instruments, Melrose Park, IL). After mixing, 1 ml of the suspensions were transferred to 9 ml of Butterfields phosphate buffer, and the Autoplate 4000 Automated Spiral Plater was used to plate the diluted *S. simulans* samples onto STA Agar; *E. coli* samples on to EMB Agar; *L. acidophilus* onto LAB Agar; and *C. perfringens* on to PER Agar. Plates of inoculated media were incubated as described above, and cfu's were enumerated.

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Statistical analysis of data: All statistical analyses were performed with GraphPad InStat® version 3.06 for Windows (GraphPad Software, San Diego, CA). One-way Analysis of Variance (ANOVA) with Dunn's Multiple Comparisons post test was performed to determine a significant difference in group means. The Mann-Whitney test was used to determine significant differences in means when only 2 values were compared. The P value for all statistical tests was ≤ 0.05 .

Results and Discussion

Skin bacterial flora recovered on PC and STA Agar:

Recovery of bacteria on PC and STA agars after washing skin in water or SA solutions exhibited similar patterns (Fig. 1 and 2). PC Agar is a nonselective medium used to enumerate several types of bacteria found in food products, while STA Agar is a selective medium used to isolate staphylococci from food and environmental samples (Zimbardo and Power, 2003). There was no significant difference in the number of bacteria recovered on either PC or STA Agar among rinsates of skin washed for 1 to 5 times in water or among samples washed in 10% SA. The decrease in the number of bacteria recovered on PC Agar from skin washed once in 20% SA and skin washed 4 or 5 times in this solution was not significantly different, although significantly fewer bacteria were recovered from skin washed 4 or 5 times 20% SA than from skin washed 2 times in this solution, however. Additionally, significantly fewer bacteria were recovered on STA Agar from skin washed 4 or 5 times in 20% SA than from skin washed once or twice in this solution. The similarities between the number of bacteria recovered on PC and STA Agars indicate that the Gram-positive cocci recovered on STA Agar may have constituted a significant portion of the bacterial population recovered on PC Agar. The repeated washing of the skin in 20% SA that was required to significantly reduce the number of bacteria recovered from the skin might indicate that the higher SA concentration is more effective in removing substances on the skin that protect bacteria from the antimicrobial activity of sanitizers. Since SA is fat soluble, the acid can become dissolved in the fat on the skin, thereby allowing the keratolytic activity of SA to remove dead layers of skin inhabited by bacteria. Additionally, SA has been reported to possess bactericidal properties that could kill some of the bacteria attached to the skin after protective fats and proteins have been removed.

Skin bacterial flora recovered on EMB Agar: The number of bacteria recovered on EMB Agar from skin after multiple washings was also dependent on the concentration of SA used to wash the skin (Fig. 3). EMB Agar is a selective medium designed to isolate Gram-negative, enteric bacteria from food and the

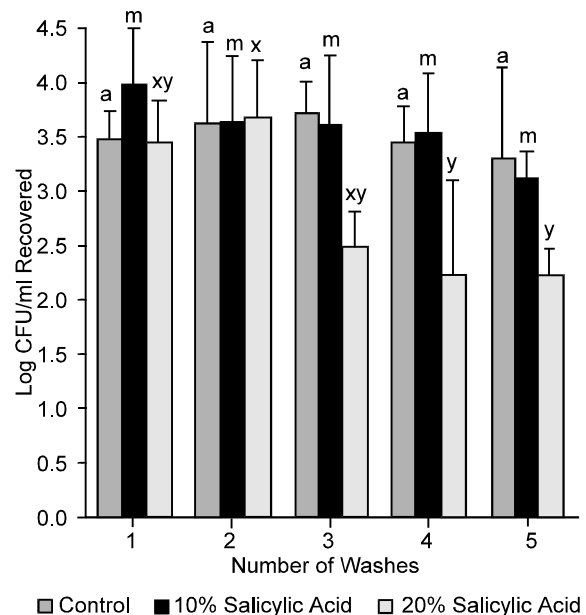


Fig. 1: Log colony-forming-units/ml recovered on Total Plate Count Agar from rinsates of broiler skin washed for 1, 2, 3, 4, or 5 times in distilled water, 10% salicylic acid, or 20% salicylic acid. Same superscripts indicate no significant differences ($p > 0.05$) in the number of bacteria recovered from skin washed in water^{ab}, 10% salicylic acid^{mn}, or 20% salicylic acid^{xy}. Values are mean \pm standard deviation. N = 5.

environment (Zimbardo and Power, 2003). Washing skin in water or 10% SA for up to 5 times did not significantly reduce the number of bacteria recovered on EMB Agar from skin of rinsates. However, approximately 1.4 and 0.80 log fewer bacteria/ml were recovered on EMB Agar from skin samples washed one time in 20% SA than from skin washed 1 time in water or 10% SA, respectively. No bacteria were recovered on this medium from skin washed 4 or 5 times in 20% SA. The greater susceptibility of Enterobacteriaceae to the antibacterial activity of SA, as compared to staphylococci on the skin, could indicate that bacteria that contaminate the carcass primarily during processing are more susceptible to the antibacterial treatments than bacteria have colonized the skin of live chickens. Gram-negative, enteric bacteria are found primarily on the feathers and in the intestines of unprocessed broilers (Cason and Hinton, 2007), but they are also found in scalding water and on the skin of picked broiler carcasses (Berrang *et al.*, 2000, Cason and Hinton, 2006, Kotula and Pandya, 1995). Enteric bacteria that contaminate the skin during processing may inhabit the water layer that covers the surface of the skin and crevices on the surface of the skin (Lillard, 1986; Thomas and McMeekin, 1980), but these bacteria may not become embedded in fats and proteins that

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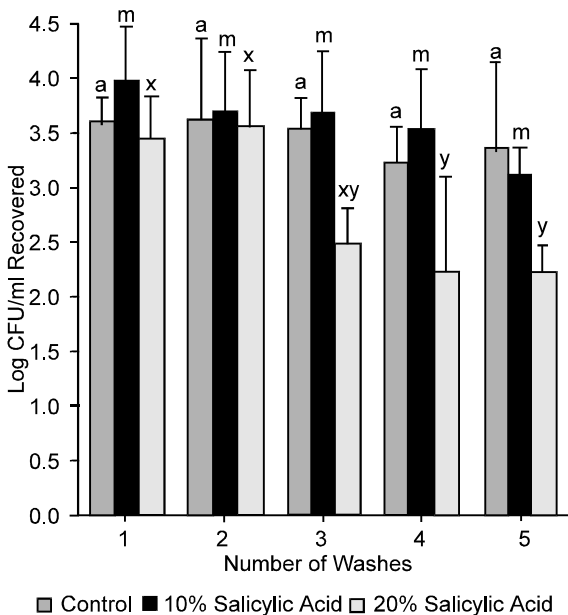


Fig. 2: Log colony-forming-units/ml recovered on *Staphylococcus* Agar from rinsates of broiler skin washed for 1, 2, 3, 4, or 5 times in distilled water, 10% salicylic acid, or 20% salicylic acid. Same superscripts indicate no significant differences ($p > 0.05$) in the number of bacteria recovered from skin washed in water^{ab}, 10% salicylic acid^{mn}, or 20% salicylic acid^{xy}. Values are mean \pm standard deviation. N = 5.

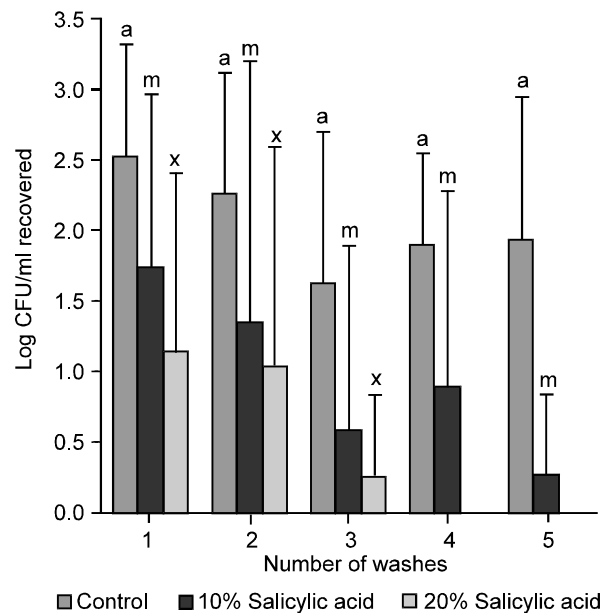


Fig. 3: Log colony-forming-units/ml recovered on Levine Eosin Methylene Blue Agar from rinsates of broiler skin washed for 1, 2, 3, 4, or 5 times in distilled water, 10% salicylic acid, or 20% salicylic acid. Same superscripts indicate no significant differences ($p > 0.05$) in the number of bacteria recovered from skin washed in water^{ab}, 10% salicylic acid^{mn}, or 20% salicylic acid^{xy}. Values are mean \pm standard deviation. N = 5.

provide protection to staphylococci on the skin. Additionally, enteric bacteria may be more susceptible to the antibacterial effects of SA.

Skin bacterial flora recovered on LAB and PER Agars:

Washing skin in SA solutions also decreased the number of bacteria recovered on LAB and PER Agars. LAB agar is a selective medium that recovers lactic acid producing bacteria (LAB) (Atlas, 1993) that may be found primarily in the crop (Hinton *et al.*, 2000), but also in other portions of the digestive tract of live broilers. The recovery of bacteria on LAB Agar was not decreased by up to 5 washings of skin in water (Fig. 4), but significantly fewer bacteria were recovered on LAB Agar from rinsates of skin washed 5 times in 10% SA than from skin washed once in this solution. Furthermore, significantly fewer bacteria were recovered from skin washed 3 times in 20% SA than from skin washed once in this solution, and no bacteria were recovered from skin washed 4 or more times in 20% SA. Since LAB are associated with alimentary tract of poultry, they may be released into processing water through the crop and cloaca during scalding and picking. In processes similar to those seen in enteric bacteria that can contaminate carcasses during scalding and defeathering, LAB may

inhabit the water layer on the skin and attach to the outer layer of skin with little protection against chemical treatments that can reduce the number of bacteria on the surface of skin.

Bacteria found in the normal microflora of the skin that are recovered on PER Agar were also susceptible to the antibacterial activity of 20% SA (Fig. 5). PER Agar is a selective medium designed to isolate and enumerate the Gram-positive anaerobes, such as *Clostridia* (Bridson, 1998). Washing skin for 5 times in water or 10% SA did not produce any significant changes in the number of bacteria recovered on PER Agar; however, no bacteria were recovered on this medium from skin washed 3 or more times in 20% SA. Since *Clostridia* are also frequently found in the intestinal tract of healthy poultry and in scald water of processing facilities (Craven, 2001), these bacteria may inhabit sites on the skin similar to those inhabited by Enterobacteriaceae and LAB where they may be exposed to the antibacterial activity of SA.

Identity of bacteria recovered from broiler skin: Many bacterial species associated with live broilers and processed broiler carcasses were identified as components of the native microflora of the skin (Table 1).

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Table 1: Identity of bacterial isolates recovered on plate count agar, *staphylococcus* agar, levine eosin methylene blue agar, lactic acid bacteria agar, and perfringens agar from rinsates of broiler chicken skin washed 5 times in distilled water or 20% salicylic acid.

Plate count Agar		Staphylococcus Agar		Levine eosin methylene blue Agar	
Distilled water	Salicylic acid 20%	Distilled water	Salicylic acid 20%	Distilled water	Salicylic acid 20%
<i>Acinetobacter baumannii</i>	<i>Staphylococcus cohnii-urealyticum</i>	<i>Staphylococcus aureus</i>	<i>Staphylococcus aureus</i>	<i>Acinetobacter baumannii</i>	None Recovered
<i>Staphylococcus aureus</i>	<i>Staphylococcus simulans</i>	<i>Staphylococcus cohnii-urealyticum</i>	<i>Staphylococcus cohnii-urealyticum</i>	<i>Escherichia coli</i>	
<i>Staphylococcus cohnii-urealyticum</i>	Unknown	<i>Staphylococcus simulans</i>	<i>Staphylococcus simulans</i>		
<i>Staphylococcus lugdensis</i>		Unknown			
Unknown					

Table 1 (continued)..

Lactic Acid Bacteria Agar		Perfringens Agar	
Distilled water	salicylic acid 20%	Distilled water	salicylic acid 20%
<i>Clostridium bifermentans</i>	None recovered	<i>Clostridium perfringens</i>	None recovered
<i>Lactobacillus plantarum</i>		<i>Lactobacillus animalis</i>	
Unknown		<i>Lactobacillus bifermentans</i>	
Unknown			

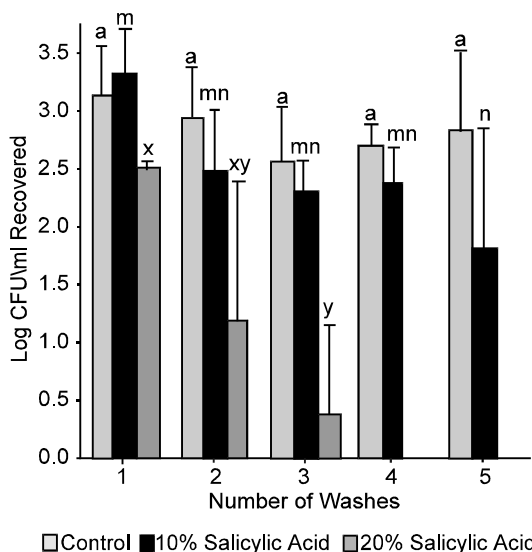


Fig. 4: Log colony-forming-units/ml recovered on Lactic Bacteria Agar from rinsates of broiler skin washed for 1, 2, 3, 4, or 5 times in distilled water, 10% salicylic acid, or 20% salicylic acid. Same superscripts indicate no significant differences ($p > 0.05$) in the number of bacteria recovered from skin washed in water^{ab}, 10% salicylic acid^{mn}, or 20% salicylic acid^{xy}. Values are mean \pm standard deviation. N = 5.

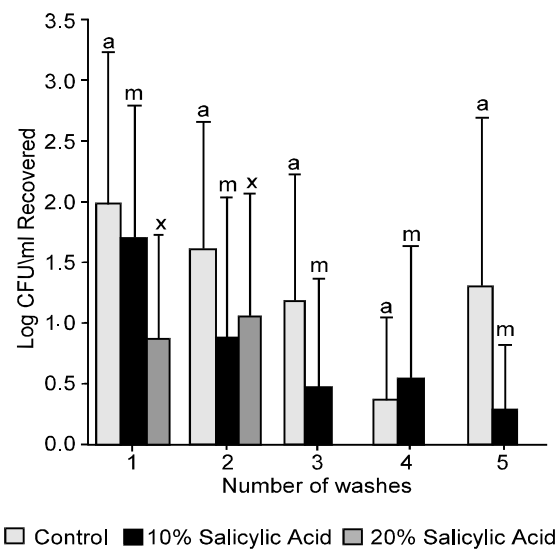


Fig. 5: Log colony-forming-units/ml recovered on Perfringens Agar from rinsates of broiler skin washed for 1, 2, 3, 4, or 5 times in distilled water, 10% salicylic acid, or 20% salicylic acid. Same superscripts indicate no significant differences ($p > 0.05$) in the number of bacteria recovered from skin washed in water^{ab}, 10% salicylic acid^{mn}, or 20% salicylic acid^{xy}. Values are mean \pm standard deviation. N = 5.

Several *Staphylococcus* sp. and *Acinetobacter baumannii* were recovered on PC Agar from skin washed in water, while only *Staphylococcus* sp. were recovered from skin washed in 20% SA. The same 3

Staphylococcus sp. were recovered on STA Agar from skin washed in water or 20% SA. *E. coli* was the primary isolate recovered on EMB from skin washed 5 times in water, but washing skin in 20% SA was able to eliminate

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Table 2: Log cfu/ml¹ bacteria recovered after mixing for 1 min in distilled water, 10% SA, or 20% SA.

Bacterial Isolate	Distilled water	10% SA	20% SA
<i>Staphylococcus Simulans</i>	4.50 ^a ± 0.26	4.19 ^a ± 0.55	0.93 ^b ± 1.27
<i>Escherichia Coli</i>	5.39 ^a ± 0.33	3.70 ^b ± 0.66	NR ²
<i>Lactobacillus Plantarum</i>	4.96 ^a ± 0.67	4.50 ^a ± 0.40	NR
<i>Clostridium Perfringens</i>	3.62 ^a ± 0.42	0.20 ^b ± 0.42	NR

Within rows, same superscripts^{ab} indicate no significant differences ($P > 0.05$) in the number of cfu/ml recovered from bacterial suspensions. ¹Values are mean ± Standard Deviation. N = 5. ²NR- None Recovered.

E. coli, as well as *A. baumannii*, found on skin samples washed in water. The LAB and PER Agar isolates consisted of species of *Lactobacillus* and *Clostridia* that are associated with the alimentary tract and feces of live poultry.

Bactericidal activity of SA solutions *in vitro*: SA exhibited bactericidal activity towards all poultry skin bacterial isolates. Significantly fewer *E. coli* and *C. perfringens* were recovered from bacterial suspensions mixed in 10% SA than from samples mixed in water, while no *E. coli*, or *Lactobacillus plantarum* were recovered from bacterial suspensions mixed in 20% SA. Furthermore, significantly fewer *S. simulans* were recovered from suspensions mixed in 20% SA than from suspensions mixed in 10% SA. The bactericidal activity of SA probably contributed to the reduction in the number of bacteria recovered from skin during washing in SA solutions. As successive washing in SA progressively removed substances from the skin that protected the bacteria from antibacterial activity of the fat soluble SA, more bacteria were exposed to the bactericide; therefore, the numbers of bacteria on the skin were reduced. Since *S. simulans* exhibited the highest level of resistance to the antibacterial activity of SA *in vitro*, this may also be one of the reasons that the staphylococci are recovered at higher levels from skin washed in SA than are other groups of bacteria.

Washing poultry skin in solutions of SA can significantly reduce the number of bacteria on the skin. Sanitizers that can penetrate the outer water layer on the skin, in addition to removing fats and other substances that protect bacteria on the skin, may be effective in reducing bacterial contamination of processed poultry products.

Acknowledgments

The authors acknowledge the technical assistance of Jerrie Barnett, Nicole Bartenfeld, Kimberly D. Ingram, Kathy Tate, and Tod Stewart.

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